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Isolation and differential expression of two isoforms of the *ROBO2/Robo2* axon guidance receptor gene in humans and mice

Ying Yue, Bärbel Grossmann, Danuta Galetzka, Ulrich Zechner, Thomas Haaf *

Institute for Human Genetics, Mainz University School of Medicine, Langenbeckstrasse 1, Building 601, 55131 Mainz, Germany

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Abstract

Expression of Robo receptor molecules is important for axon guidance across the midline of the mammalian central nervous system. Here we describe novel isoform a of human *ROBO2*, which is initially strongly expressed in the fetal human brain but thereafter only weakly expressed in adult brain and a few other tissues. The known isoform b of *ROBO2* shows a more or less ubiquitous expression pattern, suggesting diverse functional roles. The genomic structure and distinct expression patterns of *Robo2a* and *Robo2b* have been conserved in the mouse, but in contrast to human *ROBO2a* mouse *Robo2a* is also abundant in adult brain. Exons 1 and 2 of human *ROBO2a* lie in an inherently unstable DNA segment at human chromosome 3p12.3 that is associated with segmental duplications, independent chromosome rearrangements during primate evolution, and homozygous deletion and loss of heterozygosity in various human cancers. The 5′ end of mouse *Robo2a* lies in a <150-kb DNA segment of break in synteny between mouse chromosome 16C3.1 and the human genome.

Keywords: Axon guidance; Break in synteny; Chromosome 3p12.3; Evolutionary breakpoint; Fetal brain development; *ROBO2*; Segmental duplication

The *roundabout* (*Robo*) gene was first identified in a *Drosophila* screen for genes that control the decision of axons to cross the midline of the central nervous system (CNS) [1]. It encodes an axon guidance receptor that defines a novel subfamily of immunoglobulin superfamily proteins that are highly conserved from fly to human [2]. Genetic linkage and biochemical analyses revealed Slit, a large extracellular matrix protein, as ligand for Robo [3]. The *Slit* and *Robo* genes determine the neuronal patterning of commissural neurons crossing the CNS midline. In mammals, four *Robo* homologs, *Robo1* (*Dutt1*), *Robo2*, *Robo3* (*Rig1*), and *Robo4* (*magic roundabout*), have been identified. *Robo1* and *Robo2* are closely linked on human chromosome 3, mouse 16, and zebrafish 15, whereas *Robo3* and *Robo4* are linked on human 11, mouse 9, and zebrafish 10 (http://www.ensembl.org). This evolutionarily conserved genomic organization in gene pairs suggests that the *Robo* gene family arose through a series of duplication events before the split between fish and mammals more than 350 million years ago.

Mouse single mutants lacking the Robo1, Robo2, or Robo3 receptor showed commissural axon guidance defects [4,5]. *Robo1*- and *Robo2*-deficient mice also had lung [6] and kidney abnormalities [7]. In mouse, *Robo1* regulates midline crossing, while *Robo2* controls the extension of commissural axons away from the floor plate in the contralateral neural tube [4]. Mutations in human *ROBO3* prevent the motor corticospinal and dorsal somatosensory tracts from crossing the midline in the hindbrain of patients suffering from horizontal gaze palsy with progressive scoliosis and hindbrain dysplasia [8]. Homozygous deletions or loss of heterozygosity at human 3p12 is a common feature of lung carcinomas and many other malignancies. Because the critical region contains *ROBO1* and *ROBO2*, it has been concluded that these genes may not only be important for axon guidance, but also function as tumor suppressors [9].

Two isoforms of human *ROBO1*, *H-robo1* [2] and Deleted in U Twenty Twenty 1 (*DUTT1*) [9], were identified independently. Their cDNAs differ only in sequences encoding signal peptides that are cleaved in the mature protein and in the 5′

* Corresponding author. Fax: +49 6131 175690.
E-mail address: haaf@humgen.klinik.uni-mainz.de (T. Haaf).

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untranslated regions. Therefore, the longer *H-robo1* transcript is now referred to as *ROBO1a* and *DUTT1* as *ROBO1b*. Following cleavage of the signal peptide, the mature ROBO1a protein contains 32 unique amino acids at the N-terminus. RT-PCR experiments revealed that mouse *Robo1b* is more widely expressed than *Robo1a* and is the only detectable *Robo1* transcript in most adult tissues [6]. In this study, we show that similar to *Robo1*/*ROBO1*, *Robo2*/*ROBO2* encodes two differentially expressed isoforms by alternative promoter usage.

Results

Evolutionary and pathological plasticity of human chromosome 3p12.3

During our previous studies on the evolution of human chromosome 3 in primates [10–13], we found that evolutionary chromosome breakage and rearrangement are frequently associated with duplication and spreading of small segments from the breakpoint region throughout the genome. The contig of BACs RP11-73I16, RP11-58L10, and RP11-214P4 at human chromosome 3p12.3 (Fig. 1A, red squares) is 255 kb long and lies approximately 500 kb proximal to the breakpoint of an inversion that occurred in the orangutan lineage and distinguishes orangutan chromosome 2 from the orthologous human chromosome 3 (Fig. 1A, green bar). By FISH all three BACs mapped to the expected site on human chromosome 3; however, only RP11-214P4 was specific for 3p12.3. RP11-73I16 also hybridized to duplicated sequences at four subtelomeric sites and RP-58L10 weakly labeled two centromeric sites. In orangutan, the three BACs highlighted the proximal breakpoint region on orangutan chromosome 2 and many additional pericentromeric and subtelomeric regions, indicating extensive intragenomic duplications [12]. In siamang gibbon, sequences homologous to RP11-73I16, RP11-58L10, and RP11-214P4 were even more extensively amplified at essentially all subtelomeric regions and may account for several percent of the genome (Fig. 1B). Chromosome 3p12.3 not only was involved in large-scale and small-scale evolutionary chromosome rearrangements, but also is unstable in human tumor cells. The overlapping region deleted in the lung cancer cell line U2020 and the breast cancer line HCC38 contains the *Robo1* and *Robo2* genes (Fig. 1A).

Identification of a new human ROBO2 isoform

BLAST analysis of human ESTs revealed that IMAGE clone 5767984 is homologous to sequences in both RP11-58L10 and RP11-797J6, which lie 1.16 Mb apart on human chromosome 3p12.3 (Fig. 2A). Base pairs 1–388 at the 5′ end of IMAGE clone 5767984 do not show sequence similarity with any known human gene, whereas base pairs 389–832 are 95% identical with the second and third exons of *ROBO2* (Ensembl Gene No. ENSG00000185008). To study the relationship between IMAGE clone 5767984 and *ROBO2*, RT-PCR analysis was performed on human fetal brain RNA. The reverse primer (No. 1 in Table 1) for cDNA synthesis was located in exon 10 of Ensembl *ROBO2* (Fig. 2A). Forward primers were located in the first exon of IMAGE clone 5767984 (No. 2 in Table 1) and in the first exon of Ensembl *ROBO2* (No. 3 in Table 1). The

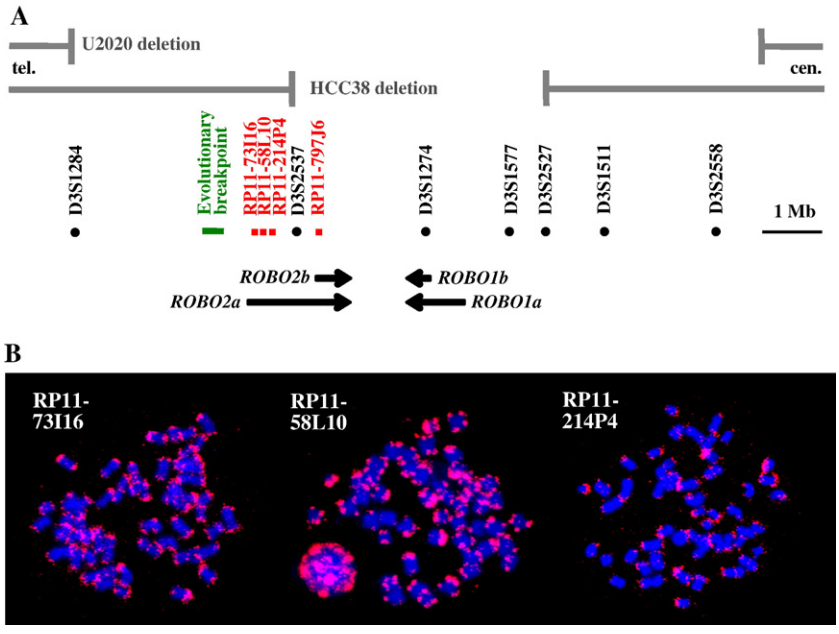

Fig. 1. (A) Map of the unstable human chromosome region 3p12.3. The U2020 and HCC38 deletion regions in lung and breast cancers are indicated by gaps between the horizontal gray lines. The evolutionary inversion breakpoint that distinguishes human chromosome 3 from orangutan 2 is located in the green DNA segment [12]. The contig of BACs RP11-73I16, RP11-58L10, and RP11-214P4 is indicated by three red squares. BAC RP11-797J6, which contains the 5′ end of *ROBO2b*, is indicated by a single red square. Black arrows indicate position and transcription direction of *ROBO1* and *ROBO2* isoforms. (B) Hybridization of human BACs RP11-73I16, RP11-58L10, and RP11-214P4 to siamang gibbon metaphase spreads.

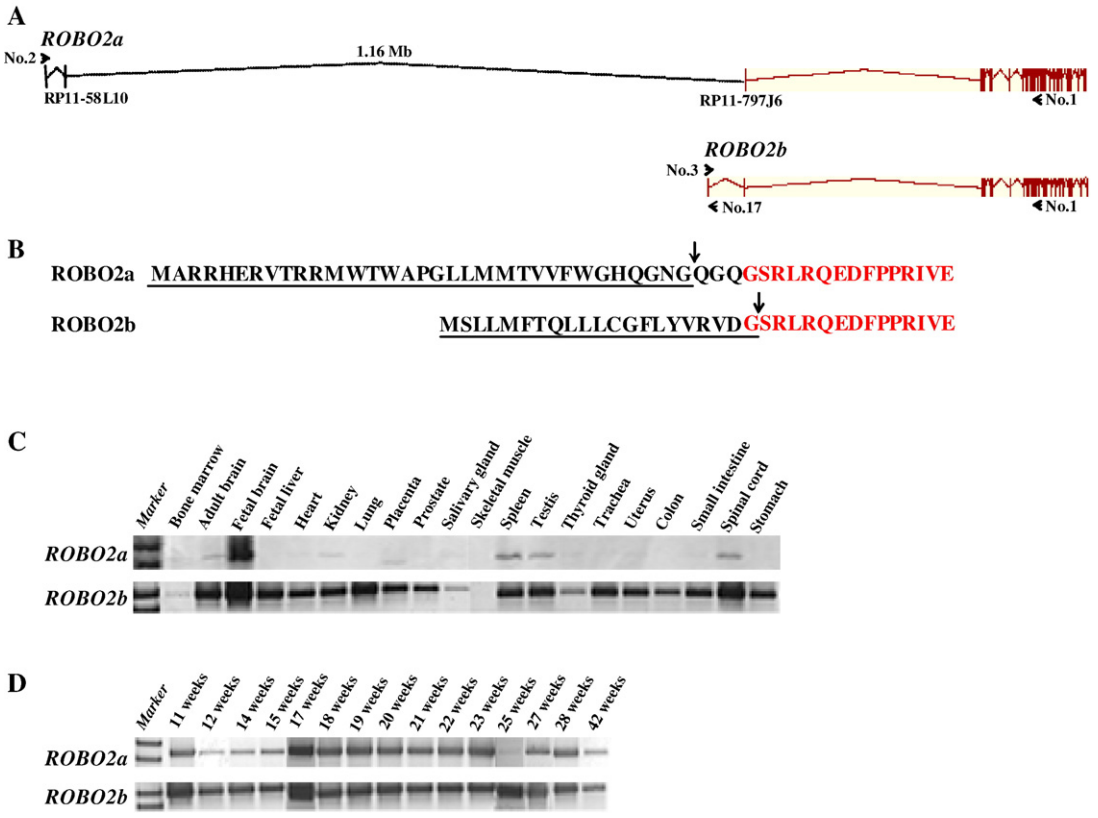

Fig. 2. (A) Genomic structure of the human *ROBO2* gene. The first two exons of isoform a are located in BAC RP11-58L10. The first two exons of isoform b are located in BAC RP11-797J6, which lies approximately 1.2 Mb proximal to RP11-58L10. Position and direction of primers (for details, see Table 1) used for amplification of the two transcripts and the 5′ end of isoform b are indicated by arrowheads. (B) Amino acid sequences of the N-terminal ends. The predicted signal peptides are underlined. Vertical arrows indicate the cleavage position. Identical amino acids in both isoforms are marked in red. (C) Transcription of *ROBO2a* and *ROBO2b* in fetal and adult human tissues, as determined by RT PCR. Isoform a is highly transcribed in fetal brain, whereas isoform b is abundant in most tissues examined. (D) *ROBO2a* and *ROBO2b* mRNA levels in fetal human brains at different weeks gestation.

resulting PCR products were cloned into the pCR 2.1-TOPO vector and sequenced. Specific products of 1698 and 1471 bp were amplified from human fetal RNA using the IMAGE clone-specific forward primer and the Ensembl *ROBO2* forward primer, respectively. Similar to the two known isoforms of *ROBO1* [6], the newly identified isoform of *ROBO2* was named *ROBO2a* and the Ensembl isoform *ROBO2b*. 5′ RACE with Primer 17 (Table 1; Fig. 2A) was used to amplify the 5′ end of *ROBO2b*. Full-length cDNA sequences were assembled from cloned cDNA fragments, IMAGE clone 5767984, and Ensembl *ROBO2* sequences. The *ROBO2a* transcript (GenBank Accession No. DQ533873) contains 27 exons and is 5920 bp long. *ROBO2b* (Accession No. DQ533874) contains 26 exons and is 6235 bp long.

Table 1
Primers for human *ROBO2* and mouse *Robo2*

Number	Sequence (5′-3′)	T_m (°C)
1	TGTAGCCACACAAGTATAAGT	58
2	CTCCCAGACAGAGAGTGG	58
3	GAGTCTGCTGATGTTTACAC	58
4	AGCTCTGTAGCTGACAGGC	60
5	GATGTGGTAGTCGCAGCTG	60
6	GCAACGATCTGATCTCTTGG	60
7	ACTTATACTTGTGTGGCTACA	60
8	CTCCAGTACAACCAGATGCT	60
9	AGCAATAGCTGGCAGACAG	58
10	ACTACAGAGCGAATGGCTG	58
11	AGCATCTGGTTGTACTGGAG	60
12	AGTGGGCAGTGGACTGCTGT	62
13	ACAGCAGTCCACTGCCCACT	60
14	GGAACTCCATGTTATAGTCTG	60
15	TACAGCAAGCCCAGCTTCC	60
16	TACACCAGCATTATCCAACC	58
17	CCGCGGGGGAAAGTCCTCCTGGCGAA	68
18	AATGCAATGGCCAGAAGACA	58

Like *ROBO1a* and *ROBO1b*, *ROBO2a* and *ROBO2b* utilize alternative exons containing different 5′ UTRs and translation start sites. The start codon of isoform a is located in exon 2 and that of isoform b in exon 1. Consequently, the two cDNA isoforms encode two protein isoforms with different N-termini. The SignalP program [14] predicted different signal peptides for the two ROBO2 protein isoforms; however, the mature proteins differ in only four amino acids at their N-terminal ends. (Fig. 2B).

Expression patterns of ROBO2 isoforms

To study the expression patterns of *ROBO2a* and *ROBO2b*, RT PCR was performed with 2 μg total RNA each

from different fetal and adult human tissues (Fig. 2C). Primer 1 (Table 1) was used to synthesize the first strand of *ROBO2* cDNA. Primer pairs 1 and 2 and 1 and 3, respectively, were used to amplify *ROBO2a* and *ROBO2b* sequences. *ROBO2b* was transcribed in all tissues examined except skeletal muscle. Transcript levels in bone marrow, salivary gland, and thyroid gland were much lower than in other tissues. Because the same cDNA templates were used for amplification of both *ROBO2a* and *ROBO2b* fragments, the ubiquitous expression of *ROBO2b* served as an internal control. In contrast to *ROBO2b*, *ROBO2a* showed a much more restricted expression pattern. The highest transcript level was observed in fetal brain, with significantly lower levels in adult brain, kidney, spleen, testis, and spinal cord. The temporal expression patterns of the two isoforms during human brain development were studied by RT-PCR analysis of human fetal brain RNAs at various (11–42) weeks of gestation (Fig. 2D). Both isoforms were detected in all developmental stages examined. This supports a role for *ROBO2* in formation of the major forebrain commissures that continues through gestation until well after birth [15].

Characterization of mouse Robo2 isoforms

Earlier chromosome painting studies revealed an association of complete orthologs of human chromosomes 3 and 21 in at least four mammalian orders. Synteny of DNA segments orthologous to human 3 and 21 has been found in all eutherian orders, including primates [16]. Fission of this ancestral chromosome 3 and 21 association occurred in Old World monkeys after the divergence of the New World monkeys [17]. Comparison of human and mouse genomic sequences revealed a contiguous segment on mouse chromosome 16C3.1 that is syntenic to human chromosomes 3p12.3 and 21q11.2. By BLAST analysis of the current human and mouse genome assemblies we localized the evolutionary fission breakpoint to an approximately 1-Mb interval on mouse chromosome 16 between the mouse Ensembl *Robo2* (at 73.7–74.5 Mb) and *Rbm11* genes (at 75.5 Mb). In humans, *ROBO2* and *RBM11* are on chromosomes 3p12.3 and 21q11.

Alignment of human *ROBO2* with the mouse genome sequence revealed a similar genomic structure in the mouse (Fig. 3A). The mouse *Robo2* isoform that occupied a larger genomic region was designated *Robo2a* and the smaller one *Robo2b*. The 5′ end of *Robo2a* is only 145 kb proximal to the 5′ end of *Rbm11*, which narrows the evolutionary fission breakpoint down to a very small DNA segment on mouse chromosome 16C3.1. Several primer pairs were designed with the mouse genomic sequence and RTPCR was performed to amplify cDNA fragments of the two mouse *Robo2* isoforms from adult brain RNA (Table 2). Full-length cDNA sequences of *Robo2a* (GenBank Accession No. DQ533875) and *Robo2b* (Accession No. DQ533876) were assembled from overlapping cDNA fragments and Ensembl *Robo2* (ENSMUSG00000052516). Similar to the situation in humans, the mouse Robo2a and Robo2b proteins use different signal peptides and their N-terminal ends differ at four amino acids (Fig. 3B).

Expression was analyzed by RTPCR using total RNA (5 μg each) from different adult mouse tissues and whole embryos at different developmental stages (Fig. 3C). Primers 1 and 18 (Table 1) amplified a 1531-bp fragment of mouse *Robo2a*, primers 1 and 3 a 1465-bp fragment of *Robo2b*. Mouse *Robo2a* RNA was detected only in adult brain and whole embryos, whereas *Robo2b* was also expressed in other adult tissues. In addition to different 5′ UTRs, mouse *Robo2* produced multiple alternative splice forms at the 3′ end. Alignment of the Ensembl *Robo2* sequence with the cDNA fragment between primers 13 and 14 (Table 2) revealed that Ensembl exon 25 (237 bp) was absent in the amplified cDNA. In addition, two different transcripts were amplified with primer pair 11 and 12. The longer transcript contained an additional 126-bp exon between Ensembl exons 20 and 21. Altogether, there are at least four variants of both mouse *Robo2a* and *Robo2b*, the longest transcript being 8339 bp and containing 29 exons (Table 3; Fig. 3D). Consequently, there may be at least eight different mouse Robo2 protein variants.

Discussion

In this study we identified a novel isoform of human *ROBO2* and mouse *Robo2*. Similar to *ROBO1/Robo1*, the transcripts of the two *ROBO2/Robo2* isoforms differ at their 5′ regions. The evolutionarily conserved genomic structure and distinct patterns of expression argue in favor of the notion that the two isoforms have different functions and are under selective pressure. Most likely, they already existed before the duplication of *Robo* genes in a vertebrate ancestor. The isoform-specific signal peptides may alter mRNA half-lives and/or cellular location. The generated *Robo2* knockout mice [4,7] that display kidney malformations and axon guidance defects are null mutants that are deficient for both isoforms. To demonstrate functional nonequivalence of the two *Robo2* isoforms, it will be necessary to generate mutations in the 5′ regions of *Robo2a* and *Robo2b*.

Importantly, *ROBO2a* is highly expressed in the developing human brain but not in adult brain or other adult tissues. Considering the function of Robo receptors in midline commissural axon guidance in vertebrates [1–5], it is plausible to assume that *ROBO2a* is important for midline crossing control in the developing human forebrain. The axon tracts in the corpus callosum, the anterior commissure, and the hippocampal commissure that connect the two cerebral hemispheres must be actively guided across the midline to their targets in the contralateral hemisphere. Pathological commissural formation in human brain development is associated with a wide spectrum (>50) of human congenital syndromes (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=omim). Mutations in at least 30 genes have been identified in patients with agenesis or hypoplasia of the corpus callosum [18]; however, so far no

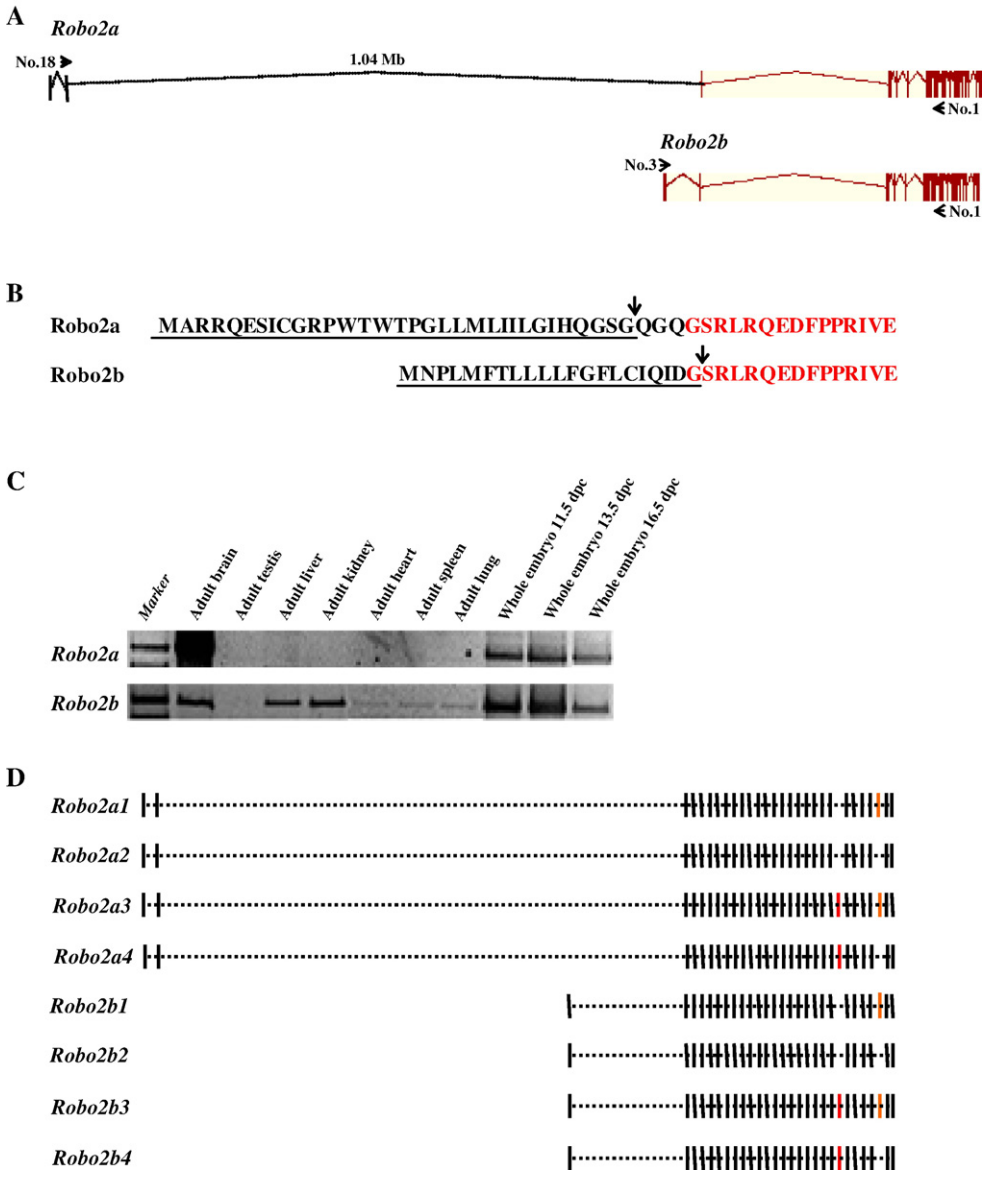

Fig. 3. (A) Genomic structure of the mouse *Robo2* gene. The first two exons of isoform a lie approximately 1 Mb distal to the first exon of isoform b. Arrowheads indicate the position and direction of primers (see Table 1) used for amplification of the two transcripts. (B) N-terminal amino acid sequences. The predicted signal peptides are underlined. Arrows indicate the cleavage position. Identical amino acids in both isoforms are marked in red. (C) Expression patterns of *Robo2a* and *Robo2b* in adult mouse tissues and whole embryos at different developmental stages. (D) Alternative splicing generates eight different mouse *Robo2* variants. Exons are indicated by vertical bars and the introns by the horizontal dotted line. The additional exon between exons 20 and 21 of Ensembl mouse *Robo2* is marked in red. Exon 25 of Ensembl mouse *Robo2* is marked in orange. Exons and intron lengths are not drawn to scale.

gene or locus for dysgenesis of forebrain commissures has been linked to chromosome 3p12.3. Because of the enormous genetic heterogeneity of anatomical and cognitive brain pathologies, patients with structural chromosome aberrations have been instrumental in positional cloning of many callosal dysgenesis and mental retardation genes.

ROBO2a was identified by FISH and in silico analyses of the evolutionary breakpoint region at human chromosome 3p12.3, which distinguishes human chromosome 3 from orangutan chromosome 2 [12] and which coincides with breaks in chromosomal synteny in the mouse, rat, and chicken genomes [11]. Fission of a conserved DNA segment syntenic to human chromosomes 3p12.3 and 21q11.2 occurred in a common ancestor of Old World monkeys and humans [16,17]. The 5′ end of mouse *Robo2a* lies in close proximity (<150 kb) of this evolutionary fission breakpoint. Expression of some genes, in particular of developmental control genes, can be influenced by regulatory elements at some distance from the transcription and promoter regions [19]. It is possible that the different expression and splicing patterns of *Robo2a*/*ROBO2a* in mice and humans are caused by evolutionary chromosome rearrangement (position effect) changing regulatory elements and/or local chromatin environment.

Intragenomic duplications of sequences homologous to BACs RP11-73I16, RP11-58L10, and RP11-214P4 from the

Table 2
Primer pairs and vectors used for cloning of mouse *Robo2*

	Forward primer[a]	Reverse primer[a]	Cloning vector
Fragment 1			
Robo2a	4	1	pCR XL-TOPO
Robo2b	3	1	pCR II-TOPO
Primer walking in fragment 1	5	6	pCR II-TOPO
Fragment 2			
Robo2	7	8	pCR II-TOPO
Primer walking in fragment 2	9	10	pCR II-TOPO
Fragment 3	11	12	pCR II-TOPO
Fragment 4			
Robo2	13	14	pCR II-TOPO
Primer walking in fragment 4	15	16	pCR II-TOPO

[a] For details, see Table 1.

3p12.3-breakpoint region occurred during primate evolution. DNA segments containing exons 1 and 2 of *ROBO2a* were extensively duplicated in the siamang and orangutan genomes and to a much lesser extent in the human genome. Biocomputational analyses of the human genome sequence revealed *ROBO2a* paralogous sequences in the centromeric regions 20p11.1 and 22q11.1. The duplicons in these regions are flanked by duplicated segments that originated from other human chromosomes. Detailed analyses of the structure and assembly of all human pericentromeric regions showed that at least 30% of the centromeric transition regions originated from euchromatic gene-containing DNA segments that were duplicatively transposed toward pericentromeric regions at a rate of six to seven events per million years during primate evolution. This process has led to the formation of at least several dozen new transcripts by exon exaptation and exon shuffling [20]. Among these new transcripts the mRNAs BC036544 at 20p11.1 and AK001299 at 22q11.1 are particularly interesting, because they are in close proximity to the duplicated *ROBO2a* exons and BC036544 has the same transcription direction. It is quite possible that the duplicated *ROBO2a* exons are fused to these transcripts, which may then provide new functions for human brain development. We propose that the widespread amplification/duplication of *ROBO2a* exons during hominoid evolution may have facilitated the evolution of new transcripts that are highly expressed in the developing fetal brain.

Materials and methods

FISH analysis on primate metaphases

Metaphase chromosomes were obtained from EBV-transformed lymphoblastoid cell lines of humans, Bornean (*Pongo pygmaeus pygmaeus*) × Sumatran orangutan (*P. pygmaeus abelii*) hybrids, and siamang gibbon (*Hylobates syndactylus*). BAC clones were selected from the Wellcome Trust Sanger Institute Ensembl contigs (http://www.ensembl.org) and obtained from the Resource Center Primary Database of the German Human Genome Project. BAC DNAs were labeled with digoxigenin-11–dUTP (Roche) by standard nick-translation and FISH-mapped on metaphase chromosomes, as described [10–13].

RT PCR and 5′-RACE PCR

Total RNAs of different human tissues (Human Total RNA Master Panel II) were obtained from Clontech. Total RNAs of human fetal brains and mouse tissues were isolated using TRIzol Reagent (Invitrogen). cDNAs were synthesized from total RNA with SuperScript III reverse transcriptase (Invitrogen). Reverse gene-specific primers were used to synthesize first-strand cDNA. Following first-strand synthesis the RNA complementary to cDNA was removed by digestion with RNase H. Target cDNA was amplified with gene-specific primer pairs by standard PCR. Only 2 μl (10%) of the first-strand reaction were used as template for subsequent PCR.

The BD SMART RACE cDNA amplification kit was used to amplify the 5′ ends of transcripts. Briefly, 5′-RACE PCR of *ROBO2b* was performed with isoform-specific primer 17 (Table 1) and Universal Primer A mix. PCR was carried out with 5 cycles of 94°C for 30 s, 72°C for 3 min; 5 cycles of 94°C for 30 s, 70°C for 30 s, 72°C for 3 min; and 25 cycles of 94°C for 30 s, 68°C for 30 s, 72°C for 3 min; with a final 10-min extension at 72°C. The PCR products were cloned into pCR 2.1-TOPO vector and sequenced.

Sequence analyses

BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) was used to align sequences to the human and mouse genomes. Synteny mapping was done with the Wellcome Trust Sanger Institute Ensembl genome browser (http://www.ensembl.org). The SignalP program [14] was used to predict the presence and location of signal peptide cleavage sites.

Acknowledgment

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Table 3
Variants of mouse *Robo2*

Isoform	Variant	Number of exons	Additional 126-bp exon[a]	Exon 25 of Ensembl *Robo2*[a]	Transcript length (bp)	Base pairs from start to stop codon/exons	Length of amino acid sequence
Mouse *Robo2a*	1	28	–	+	8213	236–4693/2–28	1485
	2	27	–	–	7940	236–4420/2–27	1394
	3	29	+	+	8339	236–4819/2–29	1527
	4	28	+	–	8066	236–4546/2–28	1436
Mouse *Robo2b*	1	27	–	+	7921	1–4402/1–27	1466
	2	26	–	–	7648	1–4128/1–26	1375
	3	28	+	+	8047	1–4527/1–28	1508
	4	27	+	–	7774	1–4254/1–27	1417

[a] "+" and "–" indicate presence or absence of a particular exon.

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